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Research Article

Purification and Characterization of the Glucose Oxidase from *Penicillium notatum*

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ABSTRACT

This study aims to purification and characterization of the glucose oxidase enzyme from *Penicillium notatum*, the enzyme was purified by ammonium sulfate precipitation (60%), dialysis and gel filtration chromatography using sephadex G-200, A trial for the purification of glucose oxidase using gel filtration technique resulted in one type of glucose oxidase with specific activity of (62.382 U/mg) with (7.385 folds) purification. the purified glucose oxidase had a maximum activity at pH = 5.5, 45 °C, glucose oxidase was stable with pH values ranging between (5 – 6) and the enzyme was maintained the activity when it incubated into (25 -35) °C for 15 minutes, analyses of the glucose oxidase for molecular weight was carried out by PAGE and SDS-PAGE electrophoresis, which revealed 78 KDa, also molecular weight of the glucose oxidase was achieved by gel filtration technique and was found 87 KDa this means that enzyme consisting of only one subunit, the K_m and V_{max} value of glucoamylase (B) were (19.6 mM, 7.5 mM/min) respectively using different concentration of glucose.

Keywords: Glucose oxidase, *Penicillium notatum*, Gel filtration.

INTRODUCTION

Glucose oxidase belongs to oxidoreductase and is also called as glucose aerodehydrogenase, Glucose oxidase (β-D glucose: oxygen-1-oxidoreductase, EC 1.1.3.4) is a fungal enzyme which catalyses the oxidation of β-D-glucose ($C_6H_{12}O_6$) to gluconolactone ($C_6H_{10}O_6$) and the concomittant reduction of molecular oxygen in hydrogen peroxide (H_2O_2). the gluconolactone then hydrolyzes spontaneously to gluconic acid ($C_6H_{12}O_7$) (Kapat, *et al*., 1998). Glucose oxidase is of considerable commercial importance due to its high specificity for glucose, its high turnover and its high stability. therefore, it has a wide range of applications (Kona, *et al*., 2001), In industry it is used to produce gluconic acid and as a source of hydrogen peroxide in food preservation (Zhu, *et al*., 2006), in pharmaceutical industry as a biosensor in diagnostic kits, it is the basis of sensors for the estimation of glucose concentration in blood, serum or plasma (Vodopivec, *et al*., 2000). glucose oxidase from *Penicillium notatum* and *Aspergillus niger* is commercially used in the food industry for the removal of glucose or oxygen to improve colour, flavour, texture and shelf life of various products (Hanft, and Koehler, 2006), the glucose oxidase from fungal origin especially ascomycetes is generally glycoprotein and consisting of two identical polypeptide chain subunits linked by disulfide bonds, the mycelia fungi *Aspergillus* such as *Aspergillus niger* and *Penicillium such as Penicillium notatum* , *Penicillium variabile* serve as industrial producers of glucose oxidase . The enzyme from *Aspergillus niger* is considered to be an intracellular enzyme, while the enzyme from *Penicillium* sp. has been generally regarded as an extracellular enzyme (Kona *et al*., 2001), it is generally accepted that the suitability of an enzyme for practical purposes depends on its thermal stability and stability in various media (Vasileva and Godjevargova, 2005).

MATERIALS AND METHODS

Inoculum preparation (Bhatti et al., 2007)

Pure culture of newly isolated *Penicillium notatum* was maintained on potato dextrose agar (PDA) slants at 4 °C, the growth (synthetic) medium (Table 1**)** was autoclaved for 15 min. after cooling the flasks, inoculum was prepared by 10 ml of sterilized distilled water was added to a sporulated 5 days old PDA slant culture, inoculum (5 ml) was added to each flask containing 150 ml of growth medium with the help of sterilized disposable syringe and flasks was incubated at 28°C on a rotary shaker at 150 rpm for 2 days.

Isolation of glucose oxidase (Shazia, et al, 2007)

At the end of fermentation, the mycelium was separated from the culture broth by filtering through a filter paper (Whatman No.1), the filtrate was centrifuged at 20 ,000 rpm for 10 min at 10° C to remove the suspended particles, the supernatant was carefully collected and stored under refrigerated conditions as to crude enzyme for further purification steps after enzyme assay.

Assay of glucose oxidase

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Table 1: The purification steps of Glucose oxidase from *Penicillium notatum.*

Purification steps	Volume (ml)	Activity (U/ml)	Total activity(U)	Protien con.(mg/ml)	Specific activity(U/mg)	Fold	Yeild %
Crude enzyme	20	6.175	123.5	0.731	8.447	1	100
Ammunium sulfate precipitation (60) %	10	4.235	42.35	0.411	10.304	1.219	0.34
Dialysis against distilled water	10	3.881	38.81	0.314	12.359	1.463	0.31
Gel filtration (First step)	5	3.214	16.07	0.082	39.195	4.640	0.13
Gel filtration (Second step)	5	2.121	10.605	0.034	62.382	7.385	0.085

Glucose oxidase activity was assayed according to (Bergmeyer, et al., 1970) by mixed 2.4 ml of the guaiacol with 0.5 ml of glucose, 0.1ml of peroxidase and 0.2 ml of the GOD solution after that, the colour developed was read each 1 min for 5 min by measuring its optical density using a spectrophotometer at 436 nm.

One enzyme activity unit (U) was defined as the amount of enzyme releasing 1 µmol of H_2O_2 from the substrate in 1 minute under standard assay conditions.

Glucose standard curve was measured according to Imran *et al* (2012).

Protein determination

Protein concentration was measured according to Bradford (1976).

Enzyme purification

Ammonium sulfate fractionation (Dennison, 2002)

The ammonium sulfate was added in different saturation ratio (20,40,60 and 80%) to reach the optimum ratio of ammonium sulfate by adding gradually the amount of salt to each 20 ml of the crude enzyme in ice bath and magnetic stirrer, centrifuge the solution for 25min at 6000 rpm. Dropped the supernatant and take the precipitate and dissolved it in 5 ml distilled water and both enzyme activity and protein content were determined for each separate fraction.

Dialysis against distilled water (Bhatti et al., 2006)

The obtained ammonium sulfate precipitate (in solution) was introduced into dialysis bag against distilled water. The obtained glucose oxidase enzyme preparation was kept in the refrigerator at 4ºC for further purification.

Gel filteration chromatography technique was measured according to El-Safey (1994) using acetate buffer (0.1 M, pH = 5), at flow rate 9 ml/hour (3ml for each fraction), the protein fractions were measured at 280 nm

Characterization of glucose oxidase (El - Gendy, 2012)

Optimal pH for enzyme activity

The glucose (substrate) was prepared with different pH ranges (4,4.5,5, 5.5 ,6 ,6.5,7, 8, 9).

Optimal pH for enzyme stability

The pure enzyme samples were incubated 1:1(V:V) in different pH ranges (4, 4.5, 5,5.5, 6, 6.5,7, 7.5, 8, 9) for 30 minutes at room temperature , then the samples were transferred freezing bath , the activity of samples were estimated using optimal pH for activity and then the relation was drawn between remaining activity and pH ranges.

Optimal temperature for enzyme activity

the glucose (substrate) was prepared in tubes, the tubes were incubated in different temperatures (10, 20, 25,30,35,40, 50) °C to 30 min for mixing, then the pure enzyme was added and incubated for 10 min to start the reaction, then the activity was estimated and the relation was drawn between enzyme activity and different temperatures.

Optimal temperature for enzyme stability

Using different temperatures at the same time (30 minutes)

The pure enzyme was incubated in tubes in different temperatures (10, 20, 25, 30, 35, 40, 50) °C to 30 min then the tubes were transferred freezing bath, then the glucose (substrate) was added the tubes were incubated in water bath for 10 minutes the activity of enzyme was estimated and the relation was drawn between remaining activity and different temperatures.

Using the same temperature at different times

The pure enzyme was incubated in tubes in 30°C to different times (10, 20, 30, 40, 50, 60) min, then the glucose (substrate) was added the tubes were incubated in water bath for 10 minutes, the activity of enzyme was estimated and the relation was drawn between remaining activity and different times.

Determination of the kinetic parameters **(K_m and V_{max})** the values of K_m and V_{max} were determined by plotting Lineweaver–Burk plot (Segel ,1976).

Etermination of the Molecular weight: include two methods:

Gel filtration technique: was measured according to Jing, *et al* (2005).

Polyacrylamide gel electrophoresis technique (Laemmli, 1970)

The molecular weight of the purified enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS - PAGE (12.5 % polyacrylamide). using Phosphorylase (97 KDa,), BSA (67 KDa, ovalbumin (43 KDa), pepsine (34 KDa), trypsine (23 KDa), lysozyme (14.4 KDa) as molecular weight standards (molecular weight marker kit S7 were from Sigma Chemical Co., USA the gel was stained with 0.25% Coomassie Brilliant Blue R- 250.

RESULTS AND DISCUSSION

Glucose oxidase purification

precipitation with ammonium sulfate

The ammonium sulfate used in different saturation ratios (20 ,40 ,60 , and 80)% , then the 60% ratio was selected as

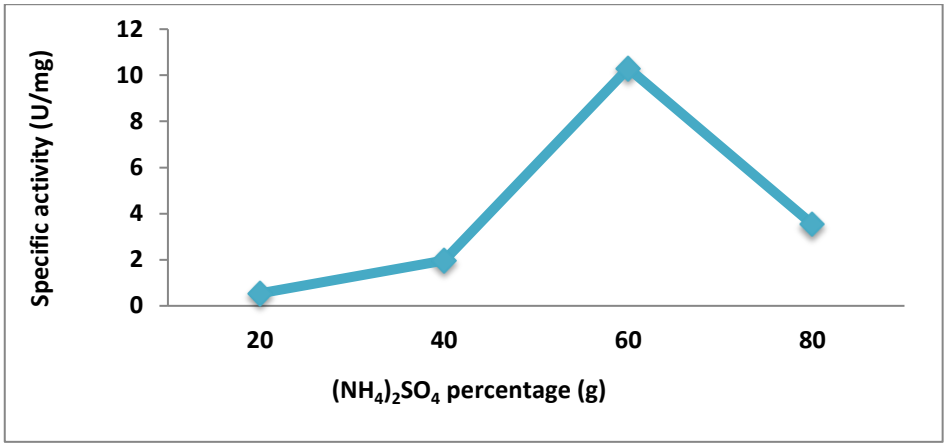

Figure 1: Effect of different Ammunium sulphate percentage on the purification of glucose oxidase from *P. notatum.*

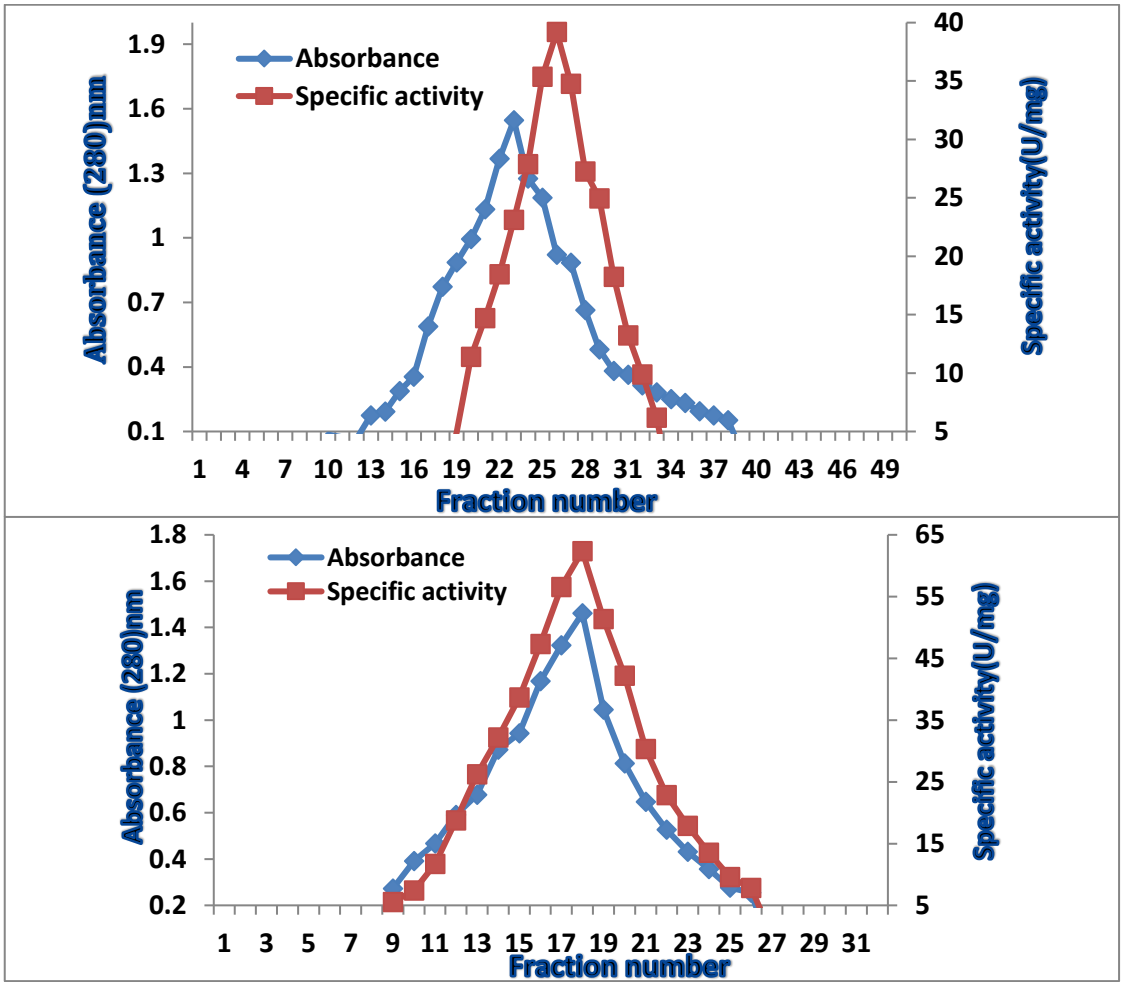

Figure 2: Gel filtration chromatography using sephadex G- 200 column with dimensions (2×40 cm) for purification the glucose oxidase from *Penicillium notatum* equilibrated with acetate buffer, (0.1 M , pH = 5) , flow rate 9 ml/hour and fraction volume 3 ml . (A) = the first step,(B) = the second step.

best ratio for precipitate the crude extract of enzyme, when the specific activity reached to (10.304 U/mg) , with a purification fold (1.219) and yield (0.34) % as shown in table (1), while the other saturation ratios (20 ,40 ,60) gave low titer of specific activity (0.542, 1.967, 3.539U/mg) respectively Figure (1). The ammonium sulfate was used in enzyme precipitation because it high soluble and cheap compared with the other salts, unaffected in pH and enzyme

stability, the concentration by ammonium sulfate depending on equilibrate the charges found in protein surface and disrupt of the water layer surrounding it, that leads to precipitate it. The other results of Simpson *et al* (2007) purified GOD from *Penicillium* sp. CBS 120262 used ammonium sulfate to precipitate GOD at the same

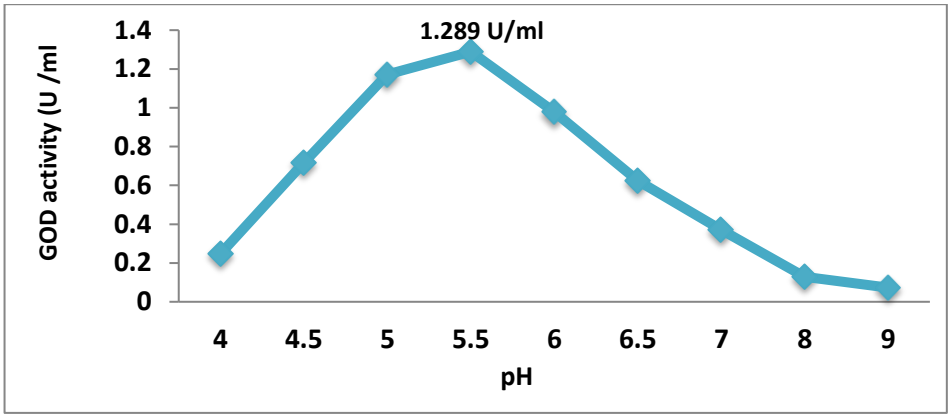

Figure 3: Effect of different pH on the activity of purified glucose oxidase from *P. notatum.*

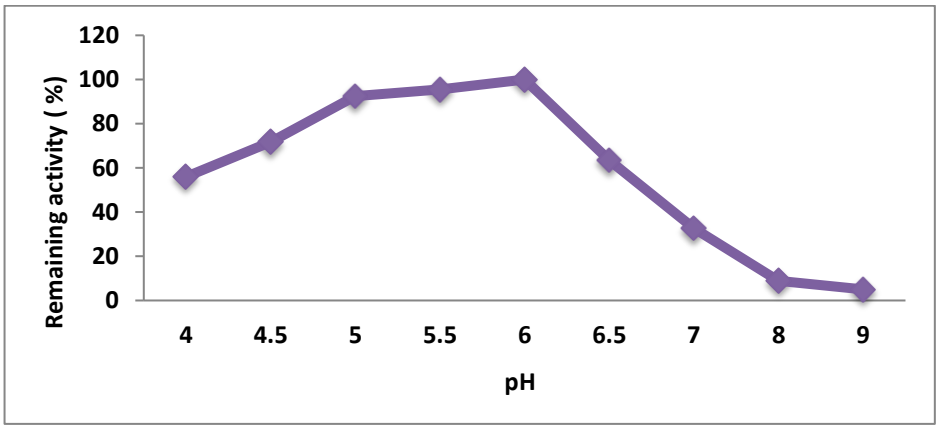

Figure 4: Effect of pH on the stability of purified glucose oxidase from *P. notatum*.

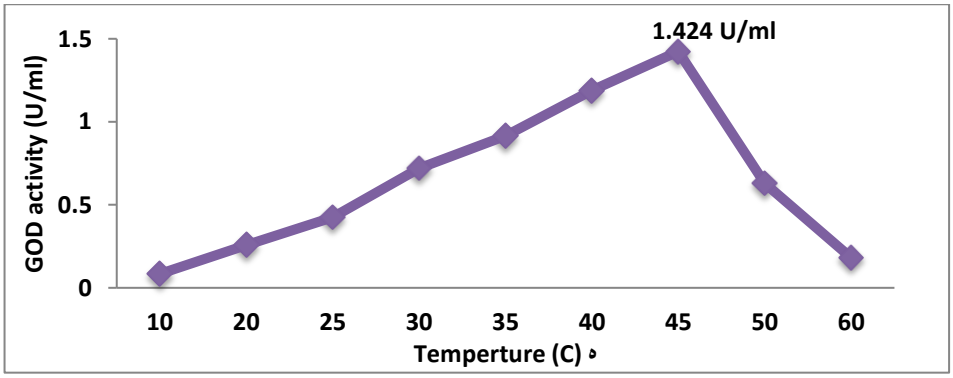

Figure 5: Effect of different temperature on the activity of purified glucose oxidase from *P. notatum* .

saturation ratio, the specific activity was (11.77 U/mg) protein with purification fold (4.9)

Dialysis against distilled water

The obtained ammonium sulfate precipitate was introduced into dialysis bag over night against distilled water, then the specific activity reached to (12.359 U/mg), with a purification fold (1.463) and yield (0.31) % as shown in table (1). Other study like, Bhatti and Saleem (2009) when they purified glucose oxidase from *Aspergillus niger* used dialysis against distilled water, the specific activity was (17.44 U/mg) protein with purification fold (1.35).

Gel filtration chromatography

The enzyme solution produced from dialysis was passed through gel filtration using (sephadex G-200) column (2×40 cm) that equilibrated with acetate buffer (0.1 M, pH =5.0), the fractions were collected from column and measured at 280 nm absorbency, one peak of protein with one peak of enzymatic activity were appeared, shown in figure (2). In the first step of gel filtration the specific activity was (39.195 U/mg) with purification fold (4.640) while in second step of gel filtration the specific activity reached (62.382 U/mg) with purification fold (7.385) shown in table (1).

Other studies recorded by, Abbas and Mahmood (2007) got specific activity (8270.8 U/mg) with purification fold (51.6) when they purified glucose oxidase from *Aspergillus niger* by using sephadex (G -150), Zia *et al* (2013) purified GOD from *Penicillium notatum* and got

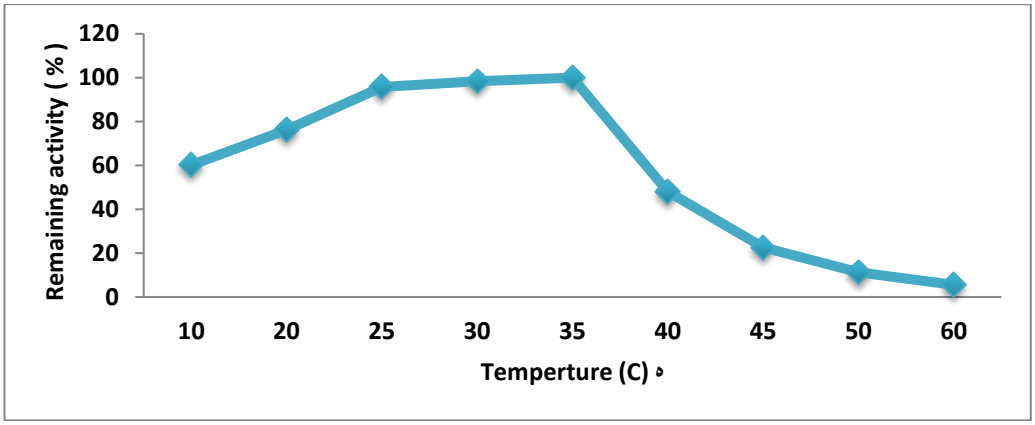

Figure 6: Effect of different incubation temperature on the stability of purified glucose oxidase from *P. notatum*

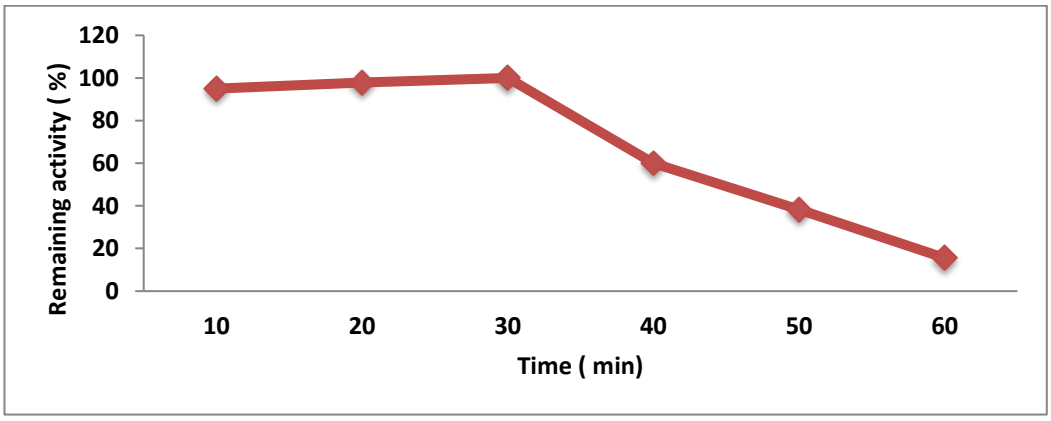

Figure 7: Effect of different incubation time on the stability of purified glucose oxidase from *P. notatum* .

Figure 8: polyacrylamide gel electrophoresis of the glucose oxidase from *Penicillium notatum* with concentration 12.5 % , lane (1) standard molecular weight markers , lane(2) crude extract enzyme , lane (3) purified glucose oxidase produced from the first step of gel filtration , lane (4) purified glucose oxidase produced from the second step of gel filtration .

specific activity (59.37 U/mg) with purification fold (4.76) by using sephadex (G-200).

Characterization of glucose oxidase

Optimum pH for enzyme activity

Figure (3) showed the increasing the activity of glucose oxidase purified from *Penicillium notatum* with increasing the pH until reach to maximum activity (1.289 U/ml) in pH = 5.5 then it began to decrease in higher pH values (0.371,0.129, 0.073 U/ml) in pH = 7, 8 ,9 respectively using glucose as a substrate of enzyme. Other studies like, Bhatti *et al* (2006) reported the highest activity of GOD purified from *Aspergillus niger* occurred at the pH = 5.5, while Anjumzia *et al* (2012) found the maximum activity of GOD purified from *Aspergillus niger* occurred at the pH= 6.

Optimum pH for enzyme stability

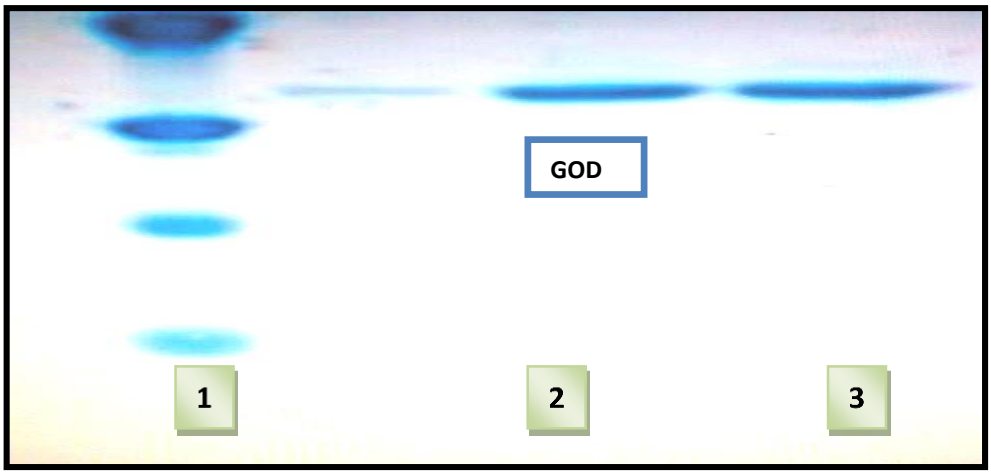

Figure 9: polyacrylamide gel electrophoresis of the glucose oxidase from *Penicillium notatum* under non - denaturing conditions with concentration 12.5 %, the electrophoresis was carried at 80 volt for 4 h . lane (1) crude extract enzyme , lane (2) purified glucose oxidase produced from the first step of gel filtration , lane (3) purified glucose oxidase produced from the second step of gel filtration.

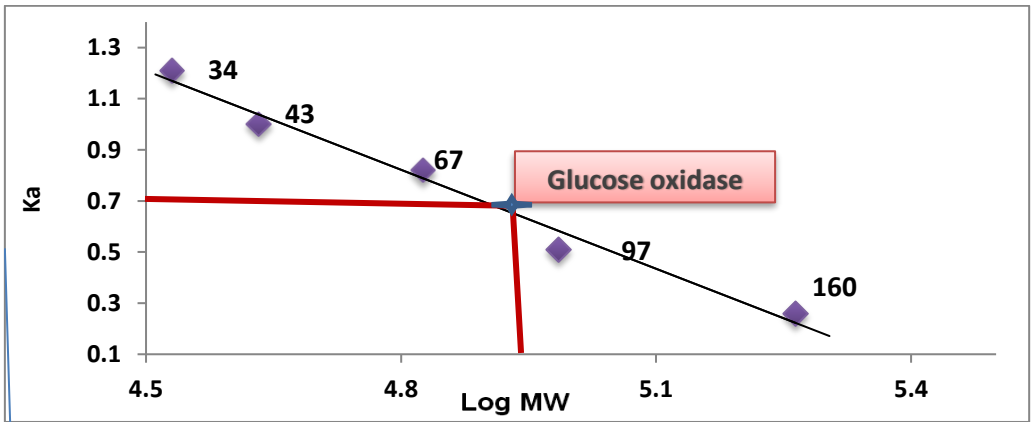

Figure 10: Determination of the molecular weight of glucose oxidase purified from *P.notatum* using gel filtration by (sephadex G- 200) column.

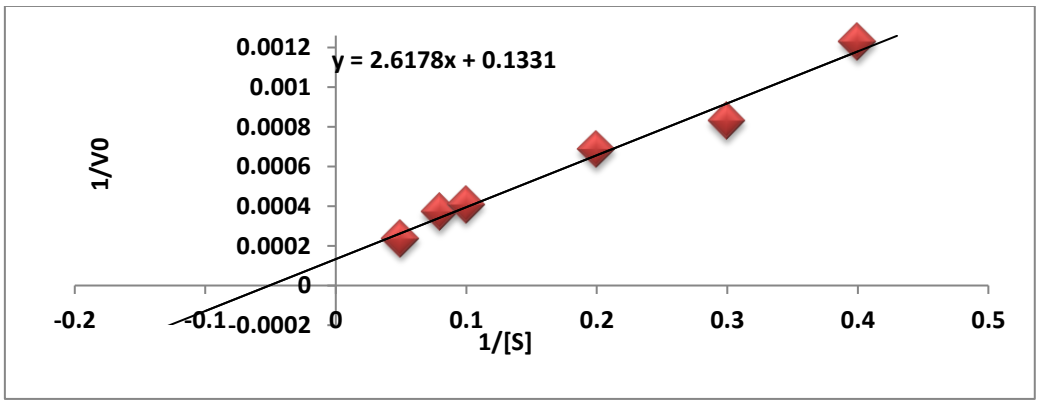

Figure 11: The relationship between substrate concentration (glucose) and velocity of glucose oxidase purified from *P. notatum*.

To study the effect of optimum pH for enzyme stability the glucose oxidase solution was incubated with different buffers pH values ranging between (4.5 - 9) for 15 minutes at room temperature, then measured the remaining activity. Figure (4) illustrated the optimum pH for GOD stability ranging between (5 - 6) and the stability was decreased in extreme acidic and alkaline pH, the enzyme was kept 72 % of its activity in pH=4.5, while the activity was decline in pH= 4, pH =8 and pH= 9 to 57 % , 9 % , 5 % respectively. Several researches pointed to the optimum pH of GOD stability, Bao *et al* (2001) found that the optimum pH of GOD stability purified from *Aspergillus*

niger G13 ranging between (4–7), while Sherbeny *et al* (2005) showed that the optimum pH of GOD stability purified from *Aspergillus niger* ranging between (4–6)

Optimum temperature of enzyme activity

To determine the optimum temperature of glucose oxidase activity purified from *Penicillium notatum* , the enzyme reaction was done in different range of temperature (10 - 60) °C , and the results shown in figure (5) increasing the activity of GOD with increasing the temperature until reached to maximum activity (1.424 U/ml) in 45 °C then it began to decrease in higher temperature values (0.629 , 0.181 U/ml) in 50, 60 °C respectively . Other studies like , Gul *et al* . (2005) found the maximum activity of GOD purified from *Penicillium* sp. was in 35 °C , while Muhammad *et al* (2007) reported the highest activity of GOD purified from *Aspergillus niger* UAF – 1 was in 45 °C

Optimum temperature of enzyme stability

Using different temperatures at the same time (15 minutes)

Figure (6) showed the results of incubation of enzyme with different temperature ranging between (10 - 60) °C for 15 minutes, the enzyme was maintained the activity when it incubated into (25 -35) °C , while keep 48% , 11% , 5 % of its activity in temperature 40 , 50 , 60 °C , respectively . Other studies recorded by , Clinton (2005) found the maximum temperature for the stability of GOD purified from *Penicillium canescens* (Tt42) ranging between (25- 30) °C for 30 min, while Singh and Verma (2013) found the optimum temperature of GOD stability purified from *Aspergillus niger* ranging between (30 -50) °C for 60 min .

Using the same temperature at different times

Figure (7) showed the results of incubation of enzyme in 45°C for different times (10 , 20 , 30 , 40 , 50 , 60) minutes , the enzyme was maintained the activity when it incubated in this temperature for (10 -30) minutes , while keep 60 % , 38 % , 15% of its activity after 40 , 50 , 60 minutes respectively , several researches pointed to the optimum temperature of GOD stability at different times , Sukhacheva *et al* (2004) found the optimum temperature of GOD stability purified from *Penicillium funiculosum* 433 was 30 °C for (20- 60) min , also Meng *et al* (2014) reported the optimum temperature of GOD stability purified from the *Aspergillus niger* was 55 °C for (10- 30) min .

Gel filtration chromatgraphy

Figure (10) showed the results of gel filtration of the glucose oxidase purified from *P.notatum*, the molecular weight of enzyme was determined by the relation was drawn between the Ka and log (molecular weight) of the each standard protein and found to be 87 kDa, this means the purified GOD have only single subunit .

Several researches pointed to molecular weight of GOD using gel filtration chromatgraphy, Aziz (1997) found the molecular weight of GOD purified from *Aspergillus niger* was 134 KDa using the gel filtration , while Zoldak *et al* (2004) showed the molecular weight of the GOD isolated from *Aspergillus niger* was 80 KDa using the same method.

Determination of K_m and V_{max} values of glucose oxidase

The values of Michalis – menten constant (K_m) and maximum velocity (V_{max}) of purified glucose oxidase from *Penicillium notatum* were estimated by using the plotting Lineweaver–Burk plot which express the relationship between reaction velocity and substrate concentration, the values of K_m and V_{max} were (19.6 mM , 7.5 mM/min) respectively using the glucose as the substrate of enzyme. Other studies like, Karmali *et al* (2004) found the values of K_m and V_{max} of the GOD purified from *Penicillium amagasakiense* were (10. 07 mM and 433.78 mg $^{-1}$ protein) respectively using the glucose as the substrate, while Rasul *et al* (2011) found the values of K_m and V_{max} of the purified glucose oxidase from *Aspergillus niger* were (2.3 mM , 10 U /ml^{-1}) respectively using the same substrate.

Determination of Molecular weight of enzyme polyacrylamide gel electrophoresis technique

In order to investigate the purity of the glucose oxidase , which was purified from *Penicillium notatum* , polyacrylamide gel electrophoresis under denaturing and non – denaturing conditions with concentration 12.5 % , the electrophoresis involve three samples , the first sample was crude enzyme extract , the second sample was the first step of gel filtration, the third sample was the second step of gel filtration, when the gel is immersed in Coomassie Brilliant Blue R- 250, several protein bands seemed with different molecular weight along the gel in crude extract sample, while one band appeared in the second and third sample, as shown in figure (8 & 9). The appearance of many protein bands along the gel is imputed to that crude extract contains large number from different proteins with different molecular weights, the second and third sample was giving only single protein band with molecular weight of approximately 78 kDa, this means that there is no contamination from other proteins. Other results recorded by, Leiter *et al* (2004) reported the molecular weight of the GOD isolated from *Penicillium chrysogenum* approximate 76 kDa, Wong *et al* (2008) found the molecular weight of the GOD isolated from *Aspergillus niger* was 80 kDa .

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